Impaired endothelial repair capacity of early endothelial progenitor cells in prehypertension: relation to endothelial dysfunction

Giannotti, G ; Doerries, C ; Mocharla, P S ; Mueller, M F ; Bahlmann, F H ; Horvàth, T ; Jiang, H ; Sorrentino, S A ; Steenken, N ; Manes, C ; Marzilli, M ; Rudolph, K L ; Lüscher, T F ; Drexler, H ; Landmesser, U

Abstract: Prehypertension is a highly frequent condition associated with an increased cardiovascular risk. Endothelial dysfunction is thought to promote the development of hypertension and vascular disease; however, underlying mechanisms remain to be further determined. The present study characterizes for the first time the in vivo endothelial repair capacity of early endothelial progenitor cells (EPCs) in patients with prehypertension/hypertension and examines its relation with endothelial function. Early EPCs were isolated from healthy subjects and newly diagnosed prehypertensive and hypertensive patients (n=52). In vivo endothelial repair capacity of EPCs was examined by transplantation into a nude mouse carotid injury model. EPC senescence was determined (RT-PCR of telomere length). NO and superoxide production of EPCs were measured using electron spin resonance spectroscopy analysis. CD34(+)/KDR(+) mononuclear cells and circulating endothelial microparticles were examined by fluorescence-activated cell sorter analysis. Endothelium-dependent and -independent vasodilations were determined by high-resolution ultrasound. In vivo endothelial repair capacity of EPCs was substantially impaired in prehypertensive/hypertensive patients as compared with healthy subjects (re-endothelialized area: 15+/-3%/13+/-2% versus 28+/-3%; P<0.05 versus healthy subjects). Senescence of EPCs in prehypertension/hypertension was substantially increased, and NO production was markedly reduced. Moreover, reduced endothelial repair capacity of early EPCs was significantly related to an accelerated senescence of early EPCs and impaired endothelial function. The present study demonstrates for the first time that in vivo endothelial repair capacity of early EPCs is reduced in patients with prehypertension and hypertension, is related to EPC senescence and impaired endothelial function, and likely represents an early event in the development of hypertension.

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Impaired Endothelial Repair Capacity of Early Endothelial Progenitor Cells in Prehypertension – Relation to Endothelial Dysfunction

Giannotti et al, Early EPC Repair Capacity in Prehypertension

Giovanna Giannotti, MD[1-3], Carola Doerries, MD[1,2], Pavani S Mocharla, BS[1]; Maja F Mueller, BS[1,2], Ferdinand H. Bahlmann, MD, PhD[4]; Tibor Horvàth, BS[2], Hong Jiang, MD[4], Sajoscha A Sorrentino, MD[2], Nora Steenken, MD[2], Costantina Manes, MD[1,2], Mario Marzilli, MD[3], K Lenhard Rudolph, MD[5], Thomas F Lüscher, MD[1], Helmut Drexler, MD[2], Ulf Landmesser, MD[1,2]

[1]Department of Cardiology, Cardiovascular Center, University Hospital Zurich, Switzerland; and Cardiovascular Research, Institute of Physiology, University of Zurich, Switzerland.

[2]Department of Cardiology and Angiology, Hannover Medical School, Hannover, Germany.

[3]Cardiac and Thoracic Department, University Hospital of Pisa, Pisa, Italy.

[4]Department of Medicine IV, University of the Saarland, Homburg/Saar, Germany.

[5]Institute of Molecular Medicine and Max Planck Research Group on Stem Cell Aging, University of Ulm, Germany.

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Address for correspondence:

Ulf Landmesser, MD
Department of Cardiology, Cardiovascular Center
University Hospital Zürich
Rämistr. 100 (C-Hof 111)
8091 Zürich
Switzerland
Tel.: +41-(0)44-255-9595
Fax: +41-(0)44-255-4401
E-mail: Ulf.Landmesser@usz.ch

ABSTRACT

Prehypertension is a highly frequent condition associated with an increased cardiovascular risk. Endothelial dysfunction is thought to promote development of hypertension and vascular disease, however, underlying mechanisms remain to be further determined. The present study characterises for the first time *in vivo* endothelial repair capacity of early endothelial progenitor cells (EPCs) in patients with prehypertension/hypertension and examines its relation with endothelial function.

Early EPCs were isolated from healthy subjects (HS) and newly diagnosed prehypertensive and hypertensive patients (n=52). *In vivo* endothelial repair capacity of EPCs was examined by transplantation into a nude mouse carotid injury model. EPC senescence was determined (RT-PCR of telomere length). Nitric oxide (NO) and superoxide production of EPCs was measured using electron spin resonance (ESR) spectroscopy analysis. CD34+/KDR+ mononuclear cells and circulating endothelial microparticles were examined by FACS analysis. Endothelium-dependent and -independent vasodilation were determined by high-resolution ultrasound. *In vivo* endothelial repair capacity of EPCs was substantially impaired in prehypertensive/hypertensive patients as compared to HS (re-endothelialized area: 15±3*/13±2* *vs.* 28±3%; $P<0.05$ *vs.* HS). Senescence of EPCs in pre/hypertension was substantially increased and NO production was markedly reduced. Moreover, reduced endothelial repair capacity of early EPCs was significantly related to an accelerated senescence of early EPCs and impaired endothelial function.

The present study demonstrates for the first time that *in vivo* endothelial repair capacity of early EPCs is reduced in patients with prehypertension and hypertension and is related to EPC senescence and impaired endothelial function, and likely represents an early event in the development of hypertension.

KEY WORDS

Endothelial function - Prehypertension - Early endothelial progenitor cells - Senescence - Nitric Oxide

INTRODUCTION

Prehypertension is a highly frequent condition affecting about 30% of the adult US population and has been defined as a systolic blood pressure range between 120-139 mmHg and/or 80-89 mmHg diastolic values.[1] [2] Prehypertension is thought to be a precursor of stage 1 hypertension, a concept that has been further supported by the observations of the TROPHY study demonstrating that treatment of prehypertension with the angiotensin-receptor blocker Candesartan prevented and postponed development of hypertension even 2 years after termination of active treatment.[3] Importantly, prehypertension is associated with a significantly increased cardiovascular risk.[4]

Endothelial dysfunction is thought to be critical in the development of vascular disease.[5] [6] Of note, Taddei et al.[7] have observed an abnormal endothelium-dependent vasodilation in the offspring of patients with essential hypertension, suggesting that endothelial dysfunction may promote the development of hypertension. Moreover, Schlaich et al.[8] have recently demonstrated that individuals with a positive family history of hypertension had an abnormal L-arginine uptake, further supporting the concept that an alteration of endothelial function may contribute to the development of essential hypertension.

Notably, recent experimental studies have suggested that endothelial progenitor cells (EPCs) promote endothelial integrit.[9] [10] [11] At present, there are in particular two populations of endothelial progenitor cells (EPCs) differentiated based on their appearance in culture, i.e. “early” EPCs appearing after 4-7 days, similar to those originally described by Asahara et al.[12], and “late” EPCs, appearing after 14-21 days.[13] [14] A beneficial effect on endothelial repair after injury has in particular been shown for early EPCs.[9] [10] [11] [15] [16] Early EPCs have also been termed “circulating angiogenic cells”, and are thought to exert their effects in particular by paracrine mechanisms.[14] [17] Furthermore, a relation between the number of in vitro formed colony forming units of early EPCs and endothelium-dependent vasodilation has been suggested.[18] However, it remains unclear whether *in vivo* endothelial repair capacity of early EPCs is altered in patients with prehypertension or hypertension and is related to an abnormal endothelium-dependent vasodilation and senescence.

In the present study, we have therefore examined *in vivo* endothelial repair capacity of early EPCs in prehypertensive and hypertensive patients as compared to healthy subjects and analysed the relationship with endothelium-dependent vasodilation and senescence. Importantly, patients included in the present study had newly diagnosed prehypertension or hypertension as their only cardiovascular risk factor. Moreover, early EPC senescence, as detected by telomere length and senescence-associated beta-galactosidase (SA-ß-Gal) staining analysis, as well as nitric oxide (NO) and superoxide production, as determined by electron spin resonance (ESR) spectroscopy analysis, were examined to understand potential mechanisms leading to an altered early EPC *in vivo* endothelial repair capacity. In addition, EPC numbers and endothelial apoptotic microparticles (CD31+/AnnexinV+ particles) were determined by FACS analysis.

METHODS

Patient Characteristics and Study Protocol: Written informed consent was obtained from all participants, and the study protocol has been approved by the local ethics committee. 130 non-smoking volunteers, aged between 40 and 70 years old, without known cardiovascular disease or ongoing pharmacological therapies were screened. Healthy subjects were included when they had no cardiovascular risk factors, a systolic blood pressure <120 mm Hg and a diastolic blood pressure <80 mm Hg (see below). Patients with prehypertension (systolic blood pressure between 120 and 139 mm Hg) or hypertension (systolic blood pressure ≥ 140 mm Hg) were included when they had no other cardiovascular risk factors. In particular, subjects with obesity (BMI > 30), hypercholesterolemia (LDL > 160 mg/dl or total cholesterol > 240 mg/dl), positive family history for cardiovascular disease, diabetes, renal impairment or known cardiovascular disease were excluded. Patients with newly diagnosed hypertension were without pharmacological therapy. Blood pressure measurements were performed according to the JNC VII/ESC 2007 recommendations, i.e. all subjects underwent at least three blood pressure measurements in two different visits, after 20-30 minutes of rest, and the measurements were spaced by 5-10 minute

intervals, on both left and right arm, in sitting and lying position. Furthermore, all participants underwent a 24-hour blood pressure measurement. Methodological details for the performed analyses, i.e. characterisation of early endothelial progenitor cells and endothelial function, are provided in the online Data Supplement (please see http://hyper.ahajournals.org).

RESULTS

Characteristics of Healthy Subjects (HS) and Patients with Prehypertension and Hypertension: The characteristics of HS and patients with prehypertension or hypertension are shown in Table 1. Notably, newly diagnosed prehypertensive and hypertensive patients were included in the present study when they did not have other cardiovascular risk factors. Moreover, no patients had to be excluded because of white coat effects, since in all enrolled patients the office blood pressure values were confirmed by 24-h blood pressure measurements.

Detection of Homing of Early EPCs to Injured Carotid Artery by FACS Analysis: Previous studies have suggested that mesenchymal stem cells have a very limited capacity to reach the carotid artery after intravenous injection, i.e. likely a low pulmonary passage.[19] In order to examine whether the intravenously injected early EPCs home to the injured carotid artery, early EPCs from healthy subjects were stained with carboxyfluorescein-diacetate-succinimidyl-ester (CFSC) and injected into the tail vein of nude mice with carotid injury ($2x10^5$ cells). fter 24 hours, the injured and the corresponding section of the uninjured contralateral carotid artery were homogenized and the number of labelled EPCs was quantified by using FACS analysis. As shown in Figure 1 A, a significant homing of labelled early EPCs was observed in the injured, but not in the uninjured carotid artery. To further examine the localisation of labelled early EPCs in the carotid artery, confocal laser scanning microscopy was performed.

Detection of Homing of Early EPCs to Injured Carotid Artery by Confocal Laser Scanning

Microscopy Analysis: Confocal laser scanning microscopy analysis was used to obtain selective images with increasing depth of the carotid arteries. TAMRA-labelled early EPCs (red signal) were detected in the endothelial repair zone of the injured carotid artery, but not in the uninjured carotid artery after injection into the tail vein of nude mice with carotid injury (Figure 1 B). Fluorescence-labelled early EPCs were localized immediately beneath the endothelial layer as indicated by serial imaging with increasing depth by using confocal laser scanning microscopy analysis of the re-endothelialized zone of the carotid artery (Figure 1 B).

***In vivo* Endothelial Repair Capacity of Early EPCs**: Transplantation of early EPCs from HS markedly accelerated endothelial repair (Figure 2 A and 2 B). Notably, *in vivo* endothelial repair capacity of early EPCs from patients with prehypertension and hypertension was markedly reduced as compared to HS (Figure 2 A). Representative photographs of repaired endothelium 3 days after transplantation of early EPCs from the three different study groups are shown in Figure 2 B. The number of early EPCs was similar after 4-day culture in the three groups (159±15 x 10^6/cm^2 in healthy subjects *vs.* 179±27 x 10^6/cm^2 in prehypertensives *vs.* 149±15 x 10^6/cm^2 in hypertensives; *P*=NS).

Endothelium-dependent and Endothelium-independent Vasodilation: Flow-dependent, endothelium-mediated vasodilation (FMD) was markedly reduced in prehypertensive patients as compared to HS (FMD: 11.6±0.7% *vs.* 8.8±0.7%, *P*=0.01; Figure 2 C). FMD was further impaired in hypertensive patients (6.5±0.5%; *P*<0.01 *vs.* prehypertensive; Figure 2 C). There was no significant difference in radial artery diameter between the groups (Table 2). Arterial blood flow measurements were performed to determine whether a reduced increase of arterial blood flow during reactive hyperemia could explain the impaired flow-dependent, endothelium-mediated vasodilation observed in patients with prehypertension or hypertension. As shown in Table 2, the values for arterial blood flow in response to reactive hyperemia in patients with prehypertension or

hypertension were not lower as compared to healthy subjects, thereby rather excluding a reduced arterial blood flow response under reactive hyperemia as a mechanism that could explain the impaired endothelium-dependent vasodilation observed in prehypertensive and hypertensive patients. Moreover, there were no differences in endothelium-independent relaxation of the radial artery between the three groups, suggesting a specifically impaired endothelium-dependent vasodilation in prehypertensive and hypertensive patients (Table 2).

Relation Between Early EPC *in vivo* Endothelial Repair Capacity and FMD: *In vivo* endothelial repair capacity of early EPCs was positively related to FMD (r=0.38, $P<0.05$; Figure 2 D), suggesting that impaired early EPC-mediated endothelial repair capacity is related to a reduced endothelium-dependent vasodilation.

Early EPC Senescence as Examined by Telomere Length and SA-ß-Gal Staining: Senescence of progenitor cells is a potential cause of altered function. We have therefore analysed markers of early EPC senescence in prehypertensive and hypertensive patients as compared to HS. Notably, a significant telomere shortening was observed in early EPCs from prehypertensive patients (HS vs. prehypertensive: 11.7±2.1 kb *vs.* 8.9±0.8 kb; $P<0.01$) and from hypertensive patients (7.5±1.1 kb; $P<0.001$ *vs.* HS; Figure 3 A) as compared to HS. As a second marker of early EPC senescence the acidic SA-ß-Gal staining of early EPCs was examined. SA-ß-Gal positive early EPCs were significantly increased in prehypertensive and hypertensive patients as compared to HS (Figure 3 B).

Moreover, there was a significant positive relationship between early EPC telomere length and early EPC *in vivo* endothelial repair capacity (r=0.31; $P<0.05$; Figure 3 C). Similarly, as evaluated by SA-ß-Gal staining, early EPC senescence was inversely related to early EPC *in vivo* endothelial repair capacity (r=-0.30; $P<0.05$; Figure 3 D), suggesting that impaired early EPC senescence is associated with an impaired *in vivo* endothelial repair capacity of early EPCs. Notably, early EPC

telomere length was inversely related to systolic blood pressure values (r=0.69; $P<0.05$; Figure 3 E). In addition, the analysis of telomerase activity in a subgroup of prehypertensive and hypertensive patients revealed a markedly reduced telomerase activity in early EPCs from these patients as compared to early EPCs from HS (HS vs. prehypertensive/hypertensive patients: 24.2±3.4% *vs.* 15.8±1.9%, n=12; $P<0.05$).

Early EPC Nitric Oxide (NO) and Superoxide Production: Early EPC NO production and oxidative stress have been suggested to play an important role for EPC repair capacity. Early EPC NO and superoxide production were therefore examined by ESR spectroscopy analysis. NO production was significantly reduced in early EPCs from prehypertensive/hypertensive patients as compared to HS (474±28 *vs.* 376±23 pmol/60 min; $P=0.01$; n=13-18; Figure 4 A). There was no significant difference in early EPC superoxide production between prehypertensive/hypertensive patients and HS (Figure 4 B).

FACS Analysis of the Number of Circulating CD34+/KDR+ Mononuclear Cells: We did not observe a significant difference in the numbers of circulating CD34/KDR double-positive mononuclear cells between newly diagnosed prehypertensive/hypertensive patients and healthy subjects (Figure 5 A).

FACS Analysis of Circulating Endothelial Apoptotic Microparticles: The number of circulating endothelial apoptotic microparticles was not significantly different between newly diagnosed prehypertensive/hypertensive patients and healthy subjects (Figure 5 B).

DISCUSSION

The present study demonstrates for the first time that *in vivo* endothelial repair capacity of early endothelial progenitor cells is substantially impaired in patients with newly diagnosed prehypertension and hypertension as their only cardiovascular risk factor. Moreover, increased senescence of early EPCs as indicated by telomere shortening and increased SA-ß-Gal-staining in prehypertensive and hypertensive patients was related to impaired EPC *in vivo* endothelial repair capacity. In addition, nitric oxide production as determined by ESR spectroscopy was substantially reduced in early EPCs derived from prehypertensive and hypertensive patients, providing a further potential underlying mechanism leading to reduced endothelial repair capacity of early EPCs in these patients. Importantly, the present study provides the first evidence that reduced *in vivo* endothelial repair capacity of early EPCs is related to impaired endothelium-dependent vasodilation. We did not observe a significant difference in the number of circulating EPCs (as assessed by CD34/KDR double positive mononuclear cells) and endothelial apoptotic microparticles in prehypertensive and hypertensive patients as compared to healthy subjects. These findings suggest that impaired *in vivo* endothelial repair capacity of early EPCs related to senescence and a reduced endothelium-dependent vasodilation represents an early event in the development of hypertension.

Endothelial dysfunction is thought to promote development of vascular disease and hypertension.[5 6 20] The present study provides novel evidence indicating that the *in vivo* endothelial repair capacity of early EPCs, as determined by transplantation of early EPCs into nude mice with vascular injury, is markedly impaired in prehypertensive and hypertensive patients without other cardiovascular risk factors. Moreover, impaired *in vivo* endothelial repair capacity of early EPCs was related to increased senescence of EPCs and an impaired endothelium-dependent vasodilation, suggesting that a reduced *in vivo* repair capacity of EPCs is an early event in the development of hypertension and vascular disease. Although these findings do not prove a cause and effect relationship, there is evidence to suggest that impaired early EPC repair capacity may contribute to endothelial

dysfunction. In a pre-clinical study, Wassmann et al. have observed that infusion of circulating early endothelial progenitor cells was able to augment endothelium-dependent vasodilation.[21] Moreover, interventions that augment early EPC function, such as statin therapy or physical exercise, are associated with improved endothelial function.[22] [23] [24] In addition, Hill et al have reported an inverse relation between the *in vitro* obtained number of EPC colony-forming units from circulating mononuclear cells and the degree of endothelial dysfunction.[18]

Importantly, Murasawa et al.[25] have recently observed that overexpression of human telomerase reverse transcriptase (TERT) in early EPCs increased their migratory activity and postnatal neovascularization capacity, suggesting that senescence and telomerase activity are important factors regulating EPC function. In the present study, we have characterized EPC senescence in prehypertensive and hypertensive patients and its relation to EPC endothelial repair capacity. Notably, both measurements of telomere length and SA-ß-Gal staining indicated an increased senescence of early EPCs from prehypertensive and hypertensive subjects, that was related to an impaired *in vivo* endothelial repair capacity of early EPCs. In addition, our ESR spectroscopy measurements revealed a reduced NO bioavailability in EPCs derived from prehypertensive/hypertensive patients. Notably, several recent studies, including work from our own group, have indicated that endothelial NO synthase is critical for EPC function, and that early EPC endothelial repair capacity is reduced as a consequence of reduced nitric oxide production in early EPCs.[10] [26] Moreover, lack of eNOS has been shown to reduce basal telomerase activity in endothelial cells, that was restored by exogenous eNOS or an NO donor, thus suggesting that a reduced NO availability may contribute to reduced telomerase activity.[27]

Notably, previous studies have indicated that mesenchymal stem cells have a very limited trans-pulmonary passage to the carotid artery after intravenous injection.[19] We have therefore performed studies to determine whether human early EPCs can be detected in the injured carotid artery after intravenous injection. After intravenous injection of labelled human early EPCs we could detect by both, i.e. FACS-analyses of homogenized carotid arteries as well as by confocal laser scanning

microscopy analyses, an increased homing of early EPCs to the injured carotid artery but not to the contralateral uninjured carotid artery. One likely explanation for the observed homing of early EPCs in the injured carotid artery after intravenous injection in the present study is that these cells are substantially smaller in size as compared to mesenchymal stem cells as studied by Fischer et al.[19] Indeed, Fischer et al. have recently observed that bone marrow-derived mononuclear cells, likely in size more similar to early EPCs, have a 30-fold higher pulmonary passage as detected in the carotid artery after intravenous injection as compared to mesenchymal stem cells.[19]

Furthermore, our confocal laser scanning microscopy analyses suggested that the injected early EPCs can be detected in the subendothelial space of the endothelial repair zone of the injured carotid artery, suggesting that these cells promote the endothelial repair process likely in particular by paracrine mechanisms. These observations are in line with a recent study by Schroeter et al.[16] that detected by fluorescence microscopy a subendothelial homing of intravenously injected early EPCs, promoting the endothelial repair response in another model of carotid injury. Moreover, these observations are consistent with a recent study by Sieveking et al. suggesting that early EPCs promote the pro-angiogenic effects largely in a paracrine fashion.[17] Therefore, the findings of the present study in context with previous findings suggest that the stimulation of the *in vivo* endothelial repair response by early EPCs is likely largely mediated by paracrine effects.

Endothelial damage is likely characterized by an imbalance of endothelial cell growth/repair and the loss of endothelial cells, i.e. by apoptosis.[28] In the present study we therefore determined the number of circulating CD34+/KDR+ and cultured early endothelial progenitor cells as well as the number of circulating endothelial apoptotic microparticles. We did not observe a significantly reduced number of cultured early EPCs or CD34+/KDR+ cells as determined by FACS analysis. However, an important difference of the present study as compared to previous studies that have examined the number of CD34+/KDR+ cells or cultured early EPCs in patients with established cardiovascular disease and cardiovascular risk factors, is that in the present study only patients with newly diagnosed prehypertension/hypertension without other cardiovascular risk factors or known

cardiovascular disease were included. Notably, if one looks closely at the results of previous studies, e.g. by Vasa et al.[29], the number of early EPCs (as determined both by number of CD34+/KDR+ cells or cultured early EPCs) was not different for patients that had only one cardiovascular risk factor as compared to control subjects, but was substantially reduced in patients with several cardiovascular risk factors. Furthermore, in the study by Vasa et al. hypertensive patients did not have a lower number of early EPCs as compared to patients without hypertension.[29] In line with these findings are observations from a recent study by Werner et al.,[30] that evaluated the prognostic value of CD34+/KDR+ cell numbers for the development of cardiovascular events in patients with coronary artery disease (CAD). In a subgroup of 432 CAD patients with arterial hypertension, there was no association between the number of CD34+/KDR+ cells and arterial hypertension. Moreover, a recent study by Delva et al.[31] examined the number of cultured early EPC in 36 patients with essential hypertension and 24 control subjects and did not report a reduced number of cultured early EPCs in hypertensive patients. However, in patients with advanced and refractory hypertension, EPC numbers were reduced in a recent study by Oliveras et al.[32] Therefore the observations of the present study in the context of previous findings are consistent with the notion that the dysfunction of early EPC is rather substantially more pronounced and begins earlier as compared to a detectable reduction of the number of circulating early EPCs in patients with hypertension. Importantly, the present study provides for the first time evidence that the *in vivo* re-endothelialisation capacity of early EPCs is already profoundly reduced in patients with prehypertension and is related to impaired endothelium-dependent vasodilation and senescence of early EPCs. Notably, whereas endothelium-dependent vasodilation was more profoundly reduced in patients with hypertension as compared to prehypertension, the endothelial repair capacity of early EPCs was already profoundly impaired in prehypertension, that is in line with the above notion, that the functional impairment of early EPCs is likely an early event in the development of hypertension.

In the present study, no increase of circulating endothelial apoptotic microparticles was observed in

prehypertensive or hypertensive patients. Notably, Preston et al.[33] have observed an increased number of circulating endothelial apoptotic microparticles in severely hypertensive patients. Werner et al. have recently reported a relation between endothelial dysfunction and the degree of endothelial cell apoptosis, as measured by circulating CD31/Annexin V positive endothelial apoptotic microparticles in patients with established coronary disease.[34] Therefore, the results of the present study together with previous observations suggest that a detectable increase of circulating endothelial apoptotic microparticles is likely occurring in a more advanced stage of hypertension or in patients with established cardiovascular disease.

Limitations of the study: Different methods are in use to isolate and culture endothelial progenitor cells.[14] At least two populations have been commonly differentiated, i.e. "early" endothelial progenitor cells and "late" outgrowth EPCs obtained after several weeks of culture as pointed out recently.[13,17] We have examined in the present study the endothelial repair capacity of early EPCs. Given that early EPCs are rather more frequent in number, they may play a particularly important role for the stimulation of endothelial repair processes. Furthermore, due to a substantial variation in the FACS-determined number of CD34+/KDR+ cells and with respect to the group sizes of the present study, conclusions with respect to the numbers of EPCs as determined by FACS analysis need to be interpreted with caution. Given that there was, however, rather a trend for increased numbers of CD34+/KDR+ cells in prehypertension/hypertension, the present data suggest that the number of these cells is likely not reduced in newly diagnosed prehypertensive/hypertensive patients without other cardiovascular risk factors or known cardiovascular disease, and that the functional impairment of early EPCs with respect to their endothelial repair capacity is likely substantially more pronounced in this early stage of the disease.

PERSPECTIVES

Endothelial dysfunction is thought to contribute to development of hypertension and atherosclerotic vascular disease, however, the underlying mechanisms are incompletely understood. The present study provides novel evidence suggesting that *in vivo* endothelial repair capacity of early endothelial progenitor cells (EPCs), likely largely mediated by paracrine effects, is markedly reduced in patients with prehypertension and hypertension, and is related to the impairment of endothelial function. These findings indicate that an impaired endothelial repair capacity of EPCs is an early event in the development of hypertension and vascular disease and raise the possibility that interventions to preserve endothelial repair capacity of EPCs, e.g. by inhibition of their accelerated senescence and reduced NO availability, may represent an attractive novel approach to stimulate endogenous endothelial repair responses and potentially to prevent endothelial dysfunction and development and progression of vascular disease.

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Disclosures:

None.

REFERENCES.

1. Chobanian AV, Bakris GL, Black HR, Cushman WC, Green LA, Izzo JL, Jr., Jones DW, Materson BJ, Oparil S, Wright JT Jr, Roccella EJ. Seventh report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure. *Hypertension* 2003; 42:1206-1252.

2. Wang Y, Wang QJ. The prevalence of prehypertension and hypertension among US adults according to the new joint national committee guidelines: new challenges of the old problem. *Arch Intern Med* 2004;164:2126-2134.

3. Julius S, Nesbitt SD, Egan BM, Weber MA, Michelson EL, Kaciroti N, Black HR, Grimm RH Jr, Messerli FH, Oparil S, Schork MA. Feasibility of treating prehypertension with an angiotensin-receptor blocker. *N Engl J Med* 2006;354:1685-1697.

4. Vasan RS, Larson MG, Leip EP, Evans JC, O'Donnell CJ, Kannel WB, Levy D. Impact of high-normal blood pressure on the risk of cardiovascular disease. *N Engl J Med* 2001;345(18):1291-7.

5. Deanfield JE, Halcox JP, Rabelink TJ. Endothelial function and dysfunction: testing and clinical relevance. *Circulation* 2007;115:1285-1295.

6. Landmesser U, Hornig B, Drexler H. Endothelial function: a critical determinant in atherosclerosis? *Circulation* 2004;109:II27-33.

7. Taddei S, Virdis A, Mattei P, Ghiadoni L, Sudano I, Salvetti A. Defective L-arginine-nitric oxide pathway in offspring of essential hypertensive patients. *Circulation* 1996;94:1298-1303.

8. Schlaich MP, Parnell MM, Ahlers BA, Finch S, Marshall T, Zhang WZ, Kaye DM. Impaired L-arginine transport and endothelial function in hypertensive and genetically predisposed normotensive subjects. *Circulation* 2004;110:3680-3686.

9. Iwakura A, Luedemann C, Shastry S, Hanley A, Kearney M, Aikawa R, Isner JM, Asahara T, Losordo DW. Estrogen-mediated, endothelial nitric oxide synthase-dependent mobilization of bone marrow-derived endothelial progenitor cells contributes to reendothelialization after arterial injury. *Circulation* 2003;108:3115-3121.

10. Sorrentino SA, Bahlmann FH, Besler C, Muller M, Schulz S, Kirchhoff N, Doerries C, Horváth T, Limbourg A, Limbourg F, Fliser D, Haller H, Drexler H, Landmesser U. Oxidant stress impairs in vivo reendothelialization capacity of endothelial progenitor cells from patients with type 2 diabetes mellitus: restoration by the peroxisome proliferator-activated receptor-gamma agonist rosiglitazone. *Circulation* 2007;116:163-173.

11. Walter DH, Rittig K, Bahlmann FH, Kirchmair R, Silver M, Murayama T, Nishimura H, Losordo DW, Asahara T, Isner JM. Statin therapy accelerates reendothelialization: a novel effect involving mobilization and incorporation of bone marrow-derived endothelial progenitor cells. *Circulation* 2002;105:3017-3024.

12. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 1997;275:964-967.

13. Shantsila E, Watson T, Tse HF, Lip GY. New insights on endothelial progenitor cell subpopulations and their angiogenic properties. *J Am Coll Cardiol* 2008;51:669-671.

14. Hirschi KK, Ingram DA, Yoder MC. Assessing identity, phenotype, and fate of endothelial progenitor cells. *Arterioscler Thromb Vasc Biol* 2008;28:1584-1595.

15. Hristov M, Zernecke A, Bidzhekov K, Liehn EA, Shagdarsuren E, Ludwig A, Weber C. Importance of CXC chemokine receptor 2 in the homing of human peripheral blood endothelial progenitor cells to sites of arterial injury. *Circ Res* 2007;100:590-597.

16. Schroeter MR, Leifheit M, Sudholt P, Heida NM, Dellas C, Rohm I, Alves F, Zientkowska M, Rafail S, Puls M, Hasenfuss G, Konstantinides S, Schäfer K. Leptin enhances the recruitment of endothelial progenitor cells into neointimal lesions after vascular injury by promoting integrin-mediated adhesion. *Circ Res* 2008;103:536-544.

17. Sieveking DP, Buckle A, Celermajer DS, Ng MK. Strikingly different angiogenic properties of endothelial progenitor cell subpopulations: insights from a novel human angiogenesis assay. *J Am Coll Cardiol* 2008;51(6):660-668.

18. Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA, Quyyumi AA, Finkel T. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N Engl J Med* 2003;348:593-600.

19. Fischer UM, Harting MT, Jimenez F, Monzon-Posadas WO, Xue H, Savitz SI, Laine GA, Cox CS Jr. Pulmonary passage is a major obstacle for intravenous stem cell delivery: the pulmonary first-pass effect. *Stem Cells Dev* 2009;18:683-692.

20. Landmesser U, Drexler H. Endothelial function and hypertension. *Curr Opin Cardiol* 2007;22:316-320.

21. Wassmann S, Werner N, Czech T, Nickenig G. Improvement of endothelial function by systemic transfusion of vascular progenitor cells. *Circ Res* 2006;99:e74-83.

22. Landmesser U, Bahlmann F, Mueller M, Spiekermann S, Kirchhoff N, Schulz S, Manes C, Fischer D, de Groot K, Fliser D, Fauler G, März W, Drexler H. Simvastatin versus ezetimibe: pleiotropic and lipid-lowering effects on endothelial function in humans. *Circulation* 2005;111:2356-2363.

23. Landmesser U, Engberding N, Bahlmann FH, Schaefer A, Wiencke A, Heineke A, Spiekermann S, Hilfiker-Kleiner D, Templin C, Kotlarz D, Mueller M, Fuchs M, Hornig B, Haller H, Drexler H. Statin-induced improvement of endothelial progenitor cell mobilization, myocardial neovascularization, left ventricular function, and survival after experimental myocardial infarction requires endothelial nitric oxide synthase. *Circulation* 2004;110:1933-1939.

24. Laufs U, Werner N, Link A, Endres M, Wassmann S, Jurgens K, Miche E, Böhm M, Nickenig G. Physical training increases endothelial progenitor cells, inhibits neointima formation, and enhances angiogenesis. *Circulation* 2004;109:220-226.

25. Murasawa S, Llevadot J, Silver M, Isner JM, Losordo DW, Asahara T. Constitutive human telomerase reverse transcriptase expression enhances regenerative properties of endothelial progenitor cells. *Circulation* 2002;106:1133-1139.

26. Aicher A, Heeschen C, Mildner-Rihm C, Urbich C, Ihling C, Technau-Ihling K, Zeiher AM,

Dimmeler S. Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells. *Nat Med* 2003;9:1370-1376.

27. Grasselli A, Nanni S, Colussi C, Aiello A, Benvenuti V, Ragone G, Moretti F, Sacchi A, Bacchetti S, Gaetano C, Capogrossi MC, Pontecorvi A, Farsetti A. Estrogen receptor-alpha and endothelial nitric oxide synthase nuclear complex regulates transcription of human telomerase. *Circ Res* 2008;103:34-42.

28. Libby P. Inflammation in atherosclerosis. *Nature* 2002;420:868-874.

29. Vasa M, Fichtlscherer S, Aicher A, Adler K, Urbich C, Martin H, Zeiher AM, Dimmeler S. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circ Res* 2001;89:E1-7.

30. Werner N, Kosiol S, Schiegl T, Ahlers P, Walenta K, Link A, Böhm M, Nickenig G. Circulating endothelial progenitor cells and cardiovascular outcomes. *N Engl J Med* 2005;353:999-1007.

31. Delva P, Degan M, Vallerio P, Arosio E, Minuz P, Amen G, Di Chio M, Lechi A. Endothelial progenitor cells in patients with essential hypertension. *J Hypertens* 2007;25:127-132.

32. Oliveras A, Soler MJ, Martinez-Estrada OM, Vázquez S, Marco-Feliu D, Vila JS, Vilaró S, Lloveras J. Endothelial progenitor cells are reduced in refractory hypertension. *J Hum Hypertens* 2008;22:183-190.

33. Preston RA, Jy W, Jimenez JJ, Mauro LM, Horstman LL, Valle M, Aime G, Ahn YS. Effects of severe hypertension on endothelial and platelet microparticles. *Hypertension* 2003;41:211-217.

34. Werner N, Wassmann S, Ahlers P, Kosiol S, Nickenig G. Circulating CD31+/annexin V+ apoptotic microparticles correlate with coronary endothelial function in patients with coronary artery disease. *Arterioscler Thromb Vasc Biol* 2006;26:112-116.

FIGURE LEGENDS

Figure 1. Detection of homing of early EPCs to the injured and uninjured carotid arteries. A. Early EPCs (2 x 10^5 cells) from healthy subjects were stained with carboxyfluorescein-diacetate-succinimidyl-ester(CFSC) and injected into the tail vein of nude mice with carotid injury. After 24 hours, the injured and the corresponding section of the contralateral uninjured carotid artery were homogenized and the number of labelled EPCs was quantified by using FACS analysis (n=3). **B.** Confocal laser scanning microscopy analysis (magnification x200) of the injured and uninjured carotid arteries. TAMRA-labelled early EPCs (red-signal) were injected into the tail vein of nude mice with carotid injury. Confocal laser scanning microscopy analysis was used to obtain selective images with increasing depth. First, the endothelial layer was visualized by whole mount CD31 immunostaining (green) of the uninjured (I) and injured (II, endothelial repair zone) carotid artery, and DAPI staining (mounting media) was used for nuclei staining (blue). Fluorescence-labelled early EPCs (TAMRA; red signal) were located beneath the endothelial layer as indicated by repeated serial imaging with increasing depth by confocal laser scanning microscopy analysis of the re-endothelialized zone of the carotid artery (III, IV). In the subendothelial layer of the endothelial repair zone of the carotid artery fluorescence-labelled early EPCs (red signal) were clearly detectable (V).

Figure 2. *In vivo* endothelial repair capacity of early EPCs and its relation to endothelium-dependent vasodilation. A. Assessment of *in vivo* endothelial repair capacity of early EPCs, expressed as the percentage of re-endothelialization on the denuded area, in mice models of carotid injury, after i.v. transplantation of early EPCs derived from healthy subjects, prehypertensive and hypertensive patients. **B.** Representative photographs of mice carotids after injury and transplantation of PBS (negative control), early EPCs from healthy, prehypertensive and hypertensive subjects. Denuded-endothelium area is stained in blue; re-endothelialized area (REA)

in white. **C.** Flow-dependent, endothelium-mediated vasodilation (FMD), expressed as the percentage change of the radial artery diameter. **D.** Relation between early EPC *in vivo* endothelial repair capacity and endothelial function, expressed as the percentage change in radial artery diameter (r=0.38; $P<0.05$).

Figure 3. Senescence of early EPCs and its relation to *in vivo* endothelial repair capacity and systolic blood pressure. **A.** Measurement of telomere length in early EPCs derived from healthy subjects, prehypertensive and hypertensive patients. **B.** SA-ß-Gal staining in early EPCs, expressed as the mean percentage of SA-ß-Gal positive cells in three random fields. **C.** Relation between early EPC telomere length and early EPC *in vivo* endothelial repair capacity. **D.** Relation between SA-ß-Gal positive early EPCs and their *in vivo* endothelial repair capacity. **E.** Relation between early EPC telomere length and systolic blood pressure.

Figure 4. Electron Spin Resonance (ESR) Spectroscopy of Nitric Oxide Availability and Oxidant Stress in Early EPCs. **A.** ESR Spectroscopy Analyses of NO production of early EPC in healthy subjects and prehypertensive/hypertensive patients; representative ESR spectra of early EPC NO production for each group are included (n=13-18). **B.** ESR Spectroscopy Analyses of early EPC Superoxide production in healthy subjects and prehypertensive/hypertensive patients (n=13-18).

Figure 5. FACS Analysis of CD34+/KDR+ Cell Number and Apoptotic Endothelial Microparticles. **A.** Percentage of circulating CD34+/KDR+ mononuclear cells in healthy, prehypertensive and hypertensive subjects. **B.** CD31+/Annexin V+ circulating apoptotic endothelial microparticles.

Table 1. Characteristics of the Study Population.

Clinical Parameters	Healthy (n=16)	Prehypertensive (n=16)	Hypertensive (n=20)	*P*-Value
Age	53±2	57±3	58±2	NS
Sex [M/F]	7/9	10/6	12/8	NS
BMI [kg/m^2]	25±0.7	24±0.8	26±0.5*	*P<0.05 vs. prehyp.
Systolic BP [mmHg]	112±2	129±2†	155±4*	*P<0.05 vs. prehyp. †P<0.05 vs. healthy
Diastolic BP [mmHg]	72±2	82±2†	93±2*	*P<0.05 vs. prehyp. †P<0.05 vs. healthy
24h Systolic BP [average; mmHg]	116±1	129±1†	146±3*	*P<0.05 vs. prehyp. †P<0.05 vs. healthy
24h Diastolic BP [average; mmHg]	73±1	81±1†	93±3*	*P<0.05 vs. prehyp. †P<0.05 vs. healthy
Fasting Glucose [mg/dl]	89±1.5	89±1.5	92±2.5	NS
HbA1c [%]	5.6±0.1	5.7±0.1	5.7±0.1	NS
LDL Cholesterol [mg/dl]	124±7	126±4	127±6	NS
HDL Cholesterol [mg/dl]	70±3	73±4	68±4	NS
Creatinin [mg/dl]	0.9±0.03	0.9±0.07	0.9±0.02	NS

Table 2. Radial Artery Diameter and Blood Flow at Baseline and during Reactive Hyperemia in Healthy Subjects, Prehypertensive and Hypertensive Patients.

Endothelium-dependent and –independent vasodilation	**Healthy**	**Prehypertensive**	**Hypertensive**	***P*-Value**
Diameter, mm				
Baseline	2.73±0.1	2.78±0.1	2.99±0.1	NS
Increase under reactive hyperemia, FMD %	11.6±0.7	8.8±0.7*	6.5±0.5*	*P*<0.05 vs. Healthy
Increase after Nitroglycerin, %	25.9±2.6	21.9±3.3	21.2±1.5	NS
Blood Flow, mL/min				
Baseline	30.6±3.6	32.7±2.6	37.4±3.0	NS
Reactive hyperemia	105±4	146±15	126±17	NS

FMD: Flow-dependent, endothelium-mediated vasodilation.